zygous missense mutations in the SAM domain of p63. *Hum Mol Genet* 10:221–9

Mills AA, Zheng B, Wang XJ *et al.* (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398: 708–13

Moretti F, Marinari B, Lo Iacono N *et al.* (2010) A regulatory feedback loop involving p63 and IRF6 links the pathogenesis of 2 genetically different human ectodermal dysplasias. *J Clin Invest* 120:1570–7

Rinne T, Bolat E, Meijer R *et al.* (2009) Spectrum of p63 mutations in a selected patient cohort affected with ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC). *Am J Med Genet A* 149A:1948–51

Rinne T, Brunner HG, van Bokhoven H (2007) p63-associated disorders. *Cell Cycle* 6: 262–8

van Bokhoven H, Jung M, Smits AP *et al.* (1999) Limb mammary syndrome: a new genetic disorder with mammary hypoplasia, ectrodactyly, and other Hand/Foot anomalies maps to human chromosome 3q27. *Am J Hum Genet* 64:538–46

Vanbokhoven H, Melino G, Candi E *et al.* (2011) p63, a story of mice and men. *J Invest Dermatol* 131:1196–207

Visinoni AF, Lisboa-Costa T, Pagnan NA *et al.* (2009) Ectodermal dysplasias: clinical and molecular review. *Am J Med Genet A* 149A: 1980–2002

Wolff S, Talos F, Palacios G *et al.* (2009) The alpha/beta carboxy-terminal domains of p63 are required for skin and limb development. New insights from the Brdm2 mouse which is not a complete p63 knockout but expresses p63 gamma-like proteins. *Cell Death Differ* 16:1108–17

Yang A, Kaghad M, Wang Y *et al.* (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2:305–16

Yang A, Schweitzer R, Sun D *et al.* (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–8

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Proinflammatory Cytokines and Chemokines at the Skin Interface during Powassan Virus Transmission

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TO THE EDITOR

Powassan virus (POWV) is an emerging North American tick-borne flavivirus transmitted to humans by infected tick bites. Ticks transmit pathogens during the complex feeding process of penetrating the skin and stay attached for several days to acquire blood. This process is facilitated by a repertoire of pharmacologically active proteins/factors in tick saliva (Ribeiro *et al.*, 2006; Kazimírová and Štibrániová, 2013). Thus, skin acts as the interface of the host–pathogen–vector interactions (Wikel, 2013). Skin provides the first line of defense against mechanical and environmental damage and infectious agents (Nestle *et al.*, 2009). In a previous study, which examined cutaneous bite-site lesions from uninfected *Ixodes scapularis* nymphs, a rapid, proinflammatory progression of the early host response was identified, culminating in the infiltration of innate immune cells by 12 hours after tick infestation (Heinze *et al.*, 2012). Successful transmission of tick-borne POWV has been shown to occur within 15 minutes of *I. scapularis* attachment (Ebel and Kramer, 2004). In addition, it was demonstrated that during early feeding time points the viral load in the tick salivary glands increases (Alekseev and Chunikhin, 1990). Therefore, the early cutaneous interactions between host immunity and initial tick-mediated immunomodulation are central to successful disease-causing agent transmission. In this study, we sought to characterize tick-induced changes in cutaneous gene expression at the early stages of attachment and feeding by POWV-infected *I. scapularis* nymphs. This will allow us to demonstrate the effect of a tick-borne virus on immune response at the tick–host interface.

In this study, we generated POWV-infected *I. scapularis* nymphs by synchronous infection (McNally *et al.*, 2012) and allowed them to feed on 6-week-old female Balb/C mice. Uninfected ticks were used as control. Each treatment group consisted of four mice, each with a capsule containing one tick. At least three out of four mice had successful tick attachment at each time point, providing us with sufficient sample sizes to perform statistical analyses. Three and six hours after tick attachment (hours post infection, h.p.i.), 4 mm mouse skin biopsies were harvested along with the feeding ticks. Ticks and skin were checked for POWV infection, and all the infected ticks/skin biopsies used in this experiment contained POWV RNA. Total RNA was extracted from each skin biopsy and cutaneous immune responses were analyzed by pathway-specific PCR arrays (Supplementary Table S1 online). In total, 456 genes were analyzed with these arrays. Relative fold differences of the immune genes were calculated as previously described (Heinze *et al.*, 2012). These data were then uploaded to ingenuity pathway analysis software for further analysis. Comparative analysis between POWV-infected and uninfected tick attachment sites at 3 and 6 h.p.i. was performed (Supplementary Table S2 online). When all significantly modulated ($P \leqslant 0.05$) host genes in the uninfected versus POWV-infected 3 h.p.i. tick-feeding sites were taken into account, there were 40 upregulated genes and 11 downregulated genes (Figure 1a). Of all significantly modulated host genes in the 6 h.p.i. uninfected versus POWV-

Abbreviations: POWV, Powassan virus; TNF, tumor necrosis factor

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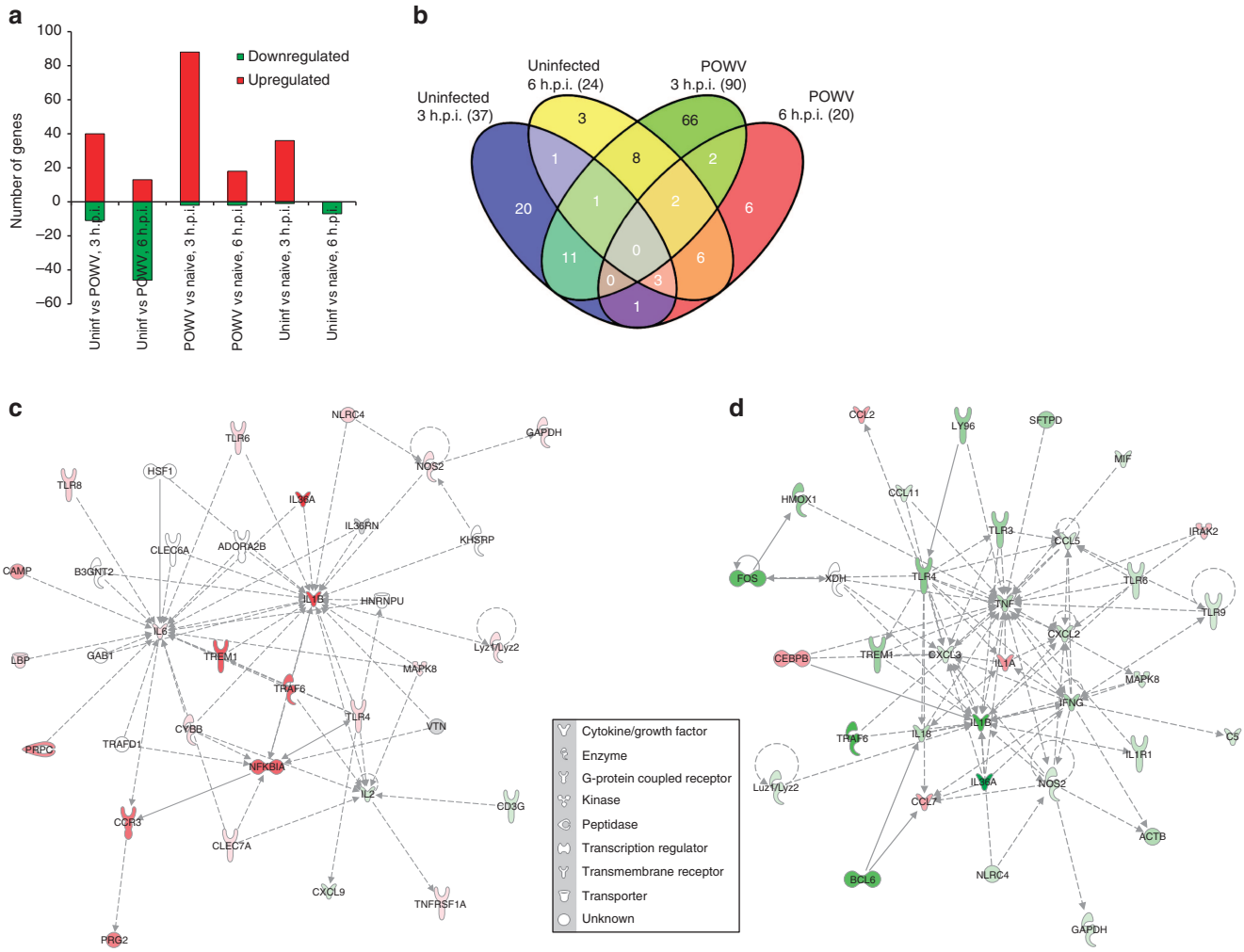

Figure 1. An overview of host gene modulation at 3 and 6 hours post infection (h.p.i.). (**a**) Number of significantly up- and downregulated genes measured at 3 and 6 h.p.i. during tick infestations of mice with *Powassan virus* (POWV)-infected *Ixodes scapularis* nymphs. (**b**) Venn diagram showing overlap of significantly modulated genes. Each time point/tick infection condition shown is versus a punch biopsy taken from naive, tick-free mice. (**c**) Top network associated with the 3 h.p.i. host immune response to the uninfected tick feeding versus POWV-infected tick feeding. (**d**) Top network associated with the 6 h.p.i. host immune response to the uninfected tick feeding versus POWV-infected tick feeding. Note: in panels **c** and **d**, red/pink represents upregulated genes; green represents downregulated genes; and gray represents unchanged or nonsignificant genes.

infected tick-feeding sites, 13 were upregulated and 46 were downregulated.

The majority of modulated genes in the 3 h.p.i. comparison were significantly upregulated and 'inflammatory response' was the top associated reaction at this time point. Several proinflammatory cytokines, such as IL1B and IL6, were significantly upregulated (Figure 2a). IL36A, which is involved in the positive regulation of IL6 production, was also upregulated. IL1B, IL6, and IL36A all influence the quantity of phagocytes and neutrophils during the inflammatory response (Fielding *et al.*, 2008; Rider *et al.*, 2011). TLR4 is another molecule, which was significantly upregulated and associated with the inflammatory response (Figure 2d). Similar to IL1B and IL6, TLR4 is linked to innate immunity. It has been shown that the TLR4 signaling pathways are involved in the innate immune response to viral infections, leading to the induction of additional proinflammatory cytokines (Okumura *et al.*, 2010). In addition, CCR3 was upregulated and it contributes to the chemotaxis of lymphocytes and eosinophils (Fahy *et al.*, 2001). To various extents, IL1B, IL6, IL36A, TLR4, and CCR3 all help establish the proinflammatory environment, which is generated by the chemotaxis of certain immune cells. These relationships are best shown in Figure 1c network, which illustrates their central role in inflammatory response regulation during POWV-infected tick feeding at 3 h.p.i.

In contrast to 3 h.p.i. comparison, the majority of significantly modulated genes at 6 h.p.i. were downregulated, including several proinflammatory cytokines associated with the inflammatory response reaction: IL1B, IL18, IFNγ, and tumor necrosis factor (TNF; Figure 2a). In Figure 1d, IFNG, IL1B, and TNF are the most connected to other molecules in this network, indicating that these three cytokines are heavily influenced by molecules that regulate the inflammatory response and cell-to-cell

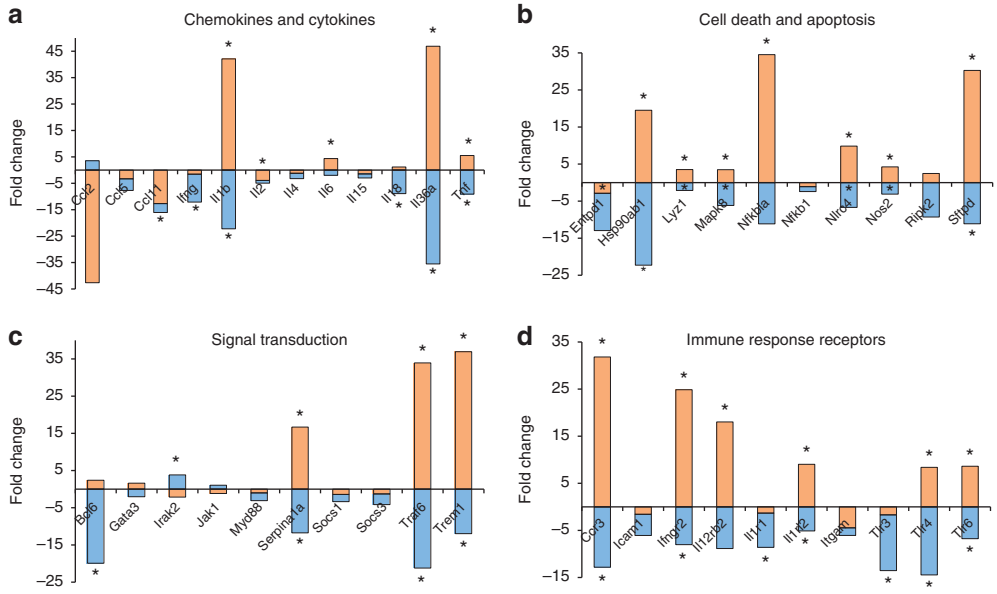

Figure 2. Changes in the host immune genes expressed in response to *Powassan virus* (POWV)-infected versus uninfected tick feeding. Genes that were modulated at both 3 hours post infection (h.p.i.) and 6 h.p.i. were compared for difference in fold-change expression levels. Genes significantly modulated at either 3 or 6 h.p.i. are marked with an asterisk. The following gene groupings were made: (**a**) chemokines and cytokines, (**b**) cell death/apoptosis, (**c**) signal transduction, (**d**) immune response receptors. A *P*-value of $\leqslant 0.05$ was considered significant and marked with an asterisk. Note: in panels **a**–**d**, orange represents genes differentially regulated at 3 h.p.i., blue represents genes differentially regulated at 6 h.p.i.

signaling during POWV-infected tick feeding at 6 h.p.i. Although the majority of genes modulated at 6 h.p.i. were downregulated, CCL2 was slightly upregulated (Figure 2a). As CCL2 has chemotactic activity for monocytes and basophils, this suggests that such immune cells are being recruited to the bite site after a POWV-infected tick has been feeding for 6 hours.

Ingenuity pathway analysis identified several significantly modulated molecules, which can either be classified under cell death and apoptosis (Figure 2b) or under signal transduction (Figure 2c). The majority of all molecules in Figure 2b and c were upregulated at 3 h.p.i. and downregulated at 6 h.p.i. Trem1 (triggering receptor expressed on myeloid cells 1) and Traf6 (TNF receptor-associated factor 6) are two molecules associated with signal transduction. In Figure 1d network, it is evident that the 3 h.p.i. upregulation of these molecules is closely tied to proinflammatory cytokines such as IL1B and IL6. Specifically, Trem1 stimulates the release of such proinflammatory cytokines, which in turn amplify the neutrophil and monocyte-mediated inflammatory response. Traf6 is a protein that facilitates signaling from the Toll/IL1 family and the TNF receptors. In response to proinflammatory cytokines, Traf6 transduces signaling in the NF-κB pathway (Wong *et al.*, 1998).

Traf6 and the NF-κB pathway are also linked with iNOS (inducible nitric oxide synthase) signaling. NOS2 (inducible nitric oxide synthase 2) is a reactive free radical, which acts as a biologic mediator in antimicrobial processes. It is inducible by a combination of lipopolysaccharide and certain cytokines such as IFNγ and TNF (Lau *et al.*, 1995). IFNGR2 and TNF were both significantly upregulated (Figure 2a and d), linking these cytokines to the host's induction of NOS2 after a POWV-infected tick has been feeding for 3 hours. This NOS2 pattern is consistent with the overall 3 h.p.i. upregulation of other molecules associated with cell death (Figure 2b).

Finally, our PCR array data demonstrated that at both 3 and 6 h.p.i., IL2 and IL4 were downregulated (Figure 2a). Therefore, our data suggest that the host's induced immune response to POWV-infected tick feeding does not have a defined Th1 or Th2 profile. This further supports our conclusion that a complex proinflammatory environment exists, with increased granulocyte recruitment, migration, and accumulation, specifically of neutrophils, at the POWV-infected tick feeding loci. Macrophages were also predicted to undergo apoptosis. Comparing POWV-infected with uninfected tick feeding loci at 6 h.p.i. predicts decreased recruitment of neutrophils and phagocytes. Our data clearly indicate that POWV-infected tick feeding recruits immune cells much earlier than the uninfected tick feeding (Figures 1 and 2). This could be directly attributed to POWV infection or changes in tick saliva secretion, or a synergistic effect of both. Further research needs to be conducted to elucidate this phenomenon. To our knowledge, this is the first report of the early cutaneous response during POWV transmission.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Alekseev A, Chunikhin S (1990) The experimental transmission of the tick-borne encephalitis virus by ixodid ticks (the mechanisms, time periods, species and sex differences). *Parazitologiia* 24:177–85

Ebel G, Kramer L (2004) Short report: duration of tick attachment required for transmission of Powassan virus by deer ticks. *Am J Trop Med Hyg* 71:268–71

Fahy O, Porte H, Sénéchal S *et al.* (2001) Chemokine-induced cutaneous inflammatory cell infiltration in a model of Hu-PBMC-SCID mice grafted with human skin. *Am J Pathol* 158:1053–63

Fielding C, McLoughlin R, McLeod L *et al.* (2008) IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. *J Immunol* 181:2189–95

Heinze D, Carmical R, Aronson J *et al.* (2012) Early immunologic events at the tick-host interface. *PLoS ONE* 7:e47301

Kazimírová M, Štibrániová I (2013) Tick salivary compounds: their role in modulation of host defences and pathogen transmission. *Front Cell Infect Microbiol* 20:43

Lau K, Nakashima O, Aalund G *et al.* (1995) TNF-alpha and IFN-gamma induce expression of nitric oxide synthase in cultured rat medullary interstitial cells. *Am J Physiol* 269: F212–7

McNally KL, Mitzel DN, Anderson JM *et al.* (2012) Differential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. *Ticks Tick Borne Dis* 3:18–26

Nestle F, Di Meglio P, Qin J *et al.* (2009) Skin immune sentinels in health and disease. *Nat Rev Immunol* 9:679–91

Okumura A, Pitha P, Yoshimura A *et al.* (2010) Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to induction of proinflammatory cytokines and SOCS1. *J Virol* 84:27–33

Ribeiro JM, Alarcon-Chaidez F, Francischetti IM *et al.* (2006) An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol* 36: 111–29

Rider P, Carmi Y, Guttman O *et al.* (2011) IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol* 187:4835–43

Wikel S (2013) Ticks and tick-borne pathogens at the cutaneous interface: host defenses, tick countermeasures, and a suitable environment for pathogen establishment. *Front Microbiol* 4:337

Wong B, Josien R, Lee S *et al.* (1998) The TRAF family of signal transducers mediates NF-κB activation by the TRANCE receptor. *J Biol Chem* 273:28355–9

Immunological and Statistical Studies of Anti-BP180 Antibodies in Paraneoplastic Pemphigus

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TO THE EDITOR

Paraneoplastic pemphigus (PNP) shows clinically intractable stomatitis and conjunctivitis with polymorphous-cutaneous lesions (Anhalt *et al.*, 1990; Hashimoto, 2001). Histopathology shows intraepidermal-acantholytic bullae and keratinocyte apoptosis (Oursler *et al.*, 1992). Most common features revealed by direct immunofluorescence (IF) are deposition of IgG to keratinocyte cell surfaces and C3 to basement membrane zone (BMZ) (Anhalt *et al.*, 1990; Hashimoto, 2001). In addition, in indirect IF, we encounter occasional reactivity with BMZ of normal skin, and more frequently with epidermal side of 1 M NaCl-split skin.

BP180 is a transmembranous collagenous protein, whose extracellular NC16a and C-terminal domains were identified as immune-dominant regions in bullous pemphigoid (BP) and mucous membrane pemphigoid, respectively (Giudice *et al.*, 1992; Matsumura *et al.*, 1996; Nie and Hashimoto, 1999; Zillikens *et al.*, 1999; Hashimoto *et al.*, 2012). Lamina lucida-type linear IgA bullous dermatosis reacts with LAD-1, truncated-extracellular domain of BP180 (Ishii *et al.*, 2008). Previous mouse model studies revealed that anti-BP180 antibodies can induce blister formation (Zillikens *et al.*, 1999), whereas the pathogenic role of BP230 is currently unclear.

Systemic study for autoantibodies to BP180 in PNP has not been performed, although a few PNP cases showed reactivity with BP180 (Preisz *et al.*, 2004). Although reactivity with BMZ in PNP may be contributed mainly by anti-BP230 antibodies, we suspected a more frequent presence of antibodies to BP180 in PNP sera.

In this study, we investigated IgG anti-BP180 antibodies in 59 PNP patients by various methods. Materials and Methods are described in Supplementary Materials online. All results of IF, immunoblotting (IB), and ELISA studies are summarized in Supplementary Table S1 online. Clinical parameters examined are shown in Supplementary Table S2 online. The results of statistical analyses are

Abbreviations: BMZ, basement membrane zone; BP, bullous pemphigoid; HaCaT, concentrated culture supernatant of HaCaT cells; IB, immunoblotting; IF, immunofluorescence; PNP, paraneoplastic pemphigus; RP, recombinant protein

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